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# “Re-educating” tumor-associated macrophages by targeting NF- $\kappa$ B

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**The nuclear factor  $\kappa$ B (NF- $\kappa$ B) signaling pathway is important in cancer-related inflammation and malignant progression. Here, we describe a new role for NF- $\kappa$ B in cancer in maintaining the immunosuppressive phenotype of tumor-associated macrophages (TAMs). We show that macrophages are polarized via interleukin (IL)-1R and MyD88 to an immunosuppressive “alternative” phenotype that requires I $\kappa$ B kinase  $\beta$ -mediated NF- $\kappa$ B activation. When NF- $\kappa$ B signaling is inhibited specifically in TAMs, they become cytotoxic to tumor cells and switch to a “classically” activated phenotype; IL-12<sup>high</sup>, major histocompatibility complex II<sup>high</sup>, but IL-10<sup>low</sup> and arginase-1<sup>low</sup>. Targeting NF- $\kappa$ B signaling in TAMs also promotes regression of advanced tumors in vivo by induction of macrophage tumoricidal activity and activation of antitumor activity through IL-12-dependent NK cell recruitment. We provide a rationale for manipulating the phenotype of the abundant macrophage population already located within the tumor microenvironment; the potential to “re-educate” the tumor-promoting macrophage population may prove an effective and novel therapeutic approach for cancer that complements existing therapies.**

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Within most human and mouse cancers there is a significant macrophage population, attracted to the tumor microenvironment by cytokines and chemokines such as CSF-1 and CCL2 (1, 2). Macrophages are plastic cells; their phenotype depends on their anatomical location and the physiological or pathological context. Classically activated macrophages (also called M1) and “alternatively” activated macrophages (M2) represent two extremes in the spectrum of the macrophage phenotype (3). Tumor-associated macrophages (TAMs) closely resemble “alternative” (M2) macrophages (4). M2 macrophages produce high amounts of IL-10 but not IL-12, express scavenger receptors, and exhibit anti-inflammatory and tissue repair functions (5). In contrast, M1 macrophages, activated by microbial products or IFN- $\gamma$ , produce large amounts of proinflammatory cytokines, express high levels of MHC molecules, and are potent killers of pathogens and tumor cells (3). Despite their intrinsic antitumor potential as both tumoricidal

cells and the major antigen-presenting cells present in tumors, ablation of macrophage function or their infiltration into experimental tumors inhibits cancer growth and metastasis (1, 6, 7). Due to the large TAM population in many tumors, an attractive therapeutic approach would be to increase their tumoricidal activity and attempt to promote antitumor immunity.

Recent studies in mouse models of colon and liver cancer have defined an important role for NF- $\kappa$ B activation in driving cancer-associated inflammation (8, 9). Our data describe an additional role for NF- $\kappa$ B in maintaining the immunosuppressive TAM phenotype. We have previously shown that malignant epithelial cells polarize macrophages to an M2-like phenotype (10, 11). Here, we show that malignant epithelial cells drive NF- $\kappa$ B activation in TAMs in a way that maintains their immunosuppressive phenotype.

The experiments described here are based on the hypothesis that NF- $\kappa$ B signaling is the central mechanism that maintains the alternative phenotype of TAMs. To investigate this we have used two different approaches to block NF- $\kappa$ B activity specifically in macrophages, both

T. Hagemann and T. Lawrence contributed equally to this work.

The online version of this article contains supplemental material.

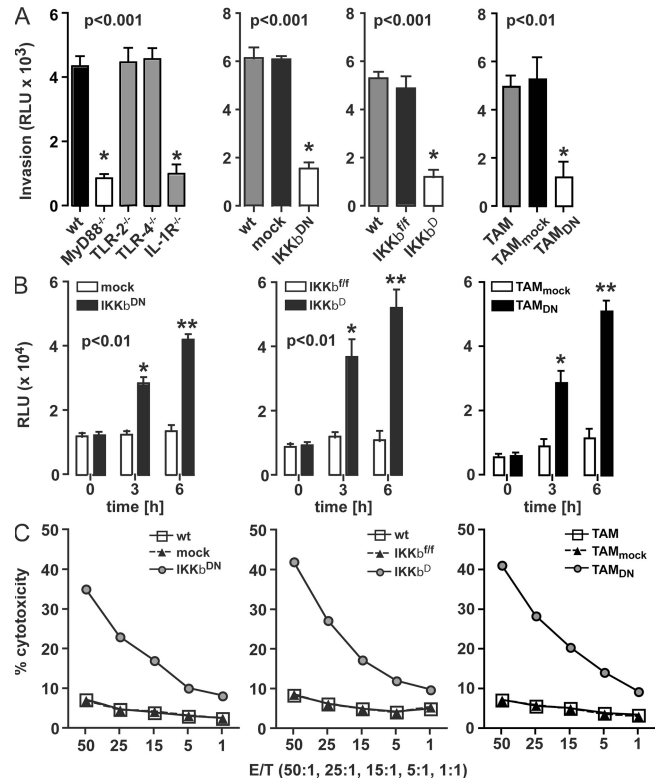
of which target I $\kappa$ B kinase (IKK) $\beta$ , the major activator of NF- $\kappa$ B. Mouse bone marrow-derived macrophages (BMDMs) were infected with a recombinant adenovirus expressing a dominant-negative inhibitor of IKK $\beta$  (Adv-IKK $\beta^{\text{DN}}$ ). IKK $\beta$  expression was also targeted in mice harboring a “floxed” *Ikk $\beta$*  allele (IKK $\beta^{\text{fl}}$ ) with adenovirus expressing Cre recombinase (Adv-Cre) to generate IKK $\beta$ -null macrophages (IKK $\beta^{\Delta}$ ) (12). Our data show that targeting IKK $\beta$  activity in BMDMs co-cultured with malignant tumor cells or TAMs isolated from established tumors reverses their tumor-promoting activity and promotes an antitumor M1 phenotype.

## RESULTS AND DISCUSSION

We previously showed that co-culture of BMDMs with human and mouse ovarian cancer cells led to increased tumor cell invasion (10, 11). In the first series of experiments described here, we investigated the macrophage signaling pathways that drive tumor cell invasion using co-cultures of BMDMs and syngeneic ID8 mouse ovarian cancer cells. Our first observation was that co-culture with macrophages deficient in MyD88 and IL-1R but not Toll-like receptor (TLR)2 or TLR4 inhibited, rather than increased, invasiveness of tumor cells in vitro (Fig. 1 A). Downstream of IL-1R and MyD88, we found that the ability of macrophages to enhance tumor cell invasiveness was dependent on NF- $\kappa$ B activation. Specific inhibition of IKK $\beta$  signaling and NF- $\kappa$ B activation by expression of IKK $\beta^{\text{DN}}$  or targeted deletion of IKK $\beta$  (IKK $\beta^{\Delta}$ ) in BMDMs prevented these cells from enhancing tumor cell invasiveness in vitro (Fig. 1 A and Fig. S1 A, which is available at <http://www.jem.org/cgi/content/full/jem.20080108/DC1>). We also isolated CD11b $^{+}$  TAMs from ID8 tumor-bearing mice and infected them in vitro with Adv-IKK $\beta^{\text{DN}}$  before co-culture with ID8 cells. As with BMDMs, inhibition of IKK $\beta$  activity in TAMs also prevented tumor cell invasion (Fig. 1 A).

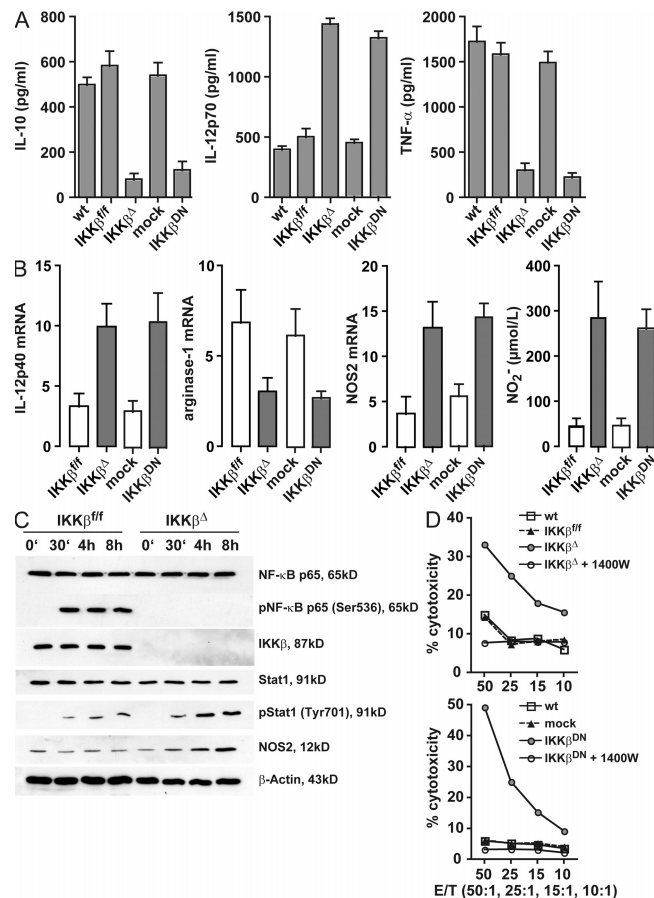
Previous literature has suggested that the mechanism by which inhibition of NF- $\kappa$ B prevented macrophage-induced tumor cell invasion might be due to decreased production of chemotactic factors by macrophages (8). But in fact we found that co-culture with IKK $\beta$ -targeted BMDMs or TAMs isolated from established tumors increased tumor cell apoptosis, as measured by caspase 3/7 activation (Fig. 1 B). In addition, inhibition of IKK $\beta$  activity in BMDMs or TAMs enhanced macrophage-mediated tumoricidal activity in vitro (Fig. 1 C).

We have previously shown that co-culture with ID8 cells polarizes macrophages to an M2-like phenotype: IL-10 $^{\text{high}}$ , IL-12 $^{\text{low}}$ , increased scavenger receptor expression, and TNF- $\alpha^{\text{high}}$  (10, 11). NF- $\kappa$ B inhibition by expression of IKK $\beta^{\text{DN}}$  or targeted deletion of IKK $\beta$  (IKK $\beta^{\Delta}$ ) in BMDMs and TAMs isolated from ID8 tumors reduced production of IL-10 and TNF- $\alpha$  upon co-culture with tumor cells in vitro (Fig. 2 A and Fig. S1 B); however, IKK $\beta$  inhibition increased production of the antitumor cytokine IL-12 (Fig. 2 A and Fig. S1 B). The IL-12 $^{\text{high}}$  IL-10 $^{\text{low}}$  phenotype of IKK $\beta$ -targeted macrophages was associated with decreased expression of arginase-1



**Figure 1. IKK $\beta$  inhibition increases macrophage tumoricidal activity.** (A) WT, MyD88 $^{-/-}$ , TLR-2 $^{-/-}$ , and TLR-4 $^{-/-}$  or mock, IKK $\beta^{\text{DN}}$  or IKK $\beta^{\text{fl}}$ , and IKK $\beta^{\Delta}$  BMDMs were co-cultured with ID8-Luc in a modified Boyden chamber without direct cell-cell contact for 72 h. In addition, CD11b $^{+}$ -selected TAMs from the ascites of ID8 tumor-bearing mice were mock (TAM $_{\text{mock}}$ ) or IKK $\beta^{\text{DN}}$  infected (TAM $_{\text{DN}}$ ). Invasion of ID8-Luc cells was assessed by luciferase activity in the lower part of the chamber. Macrophages deleted in MyD88, IL-1R, or IKK $\beta$  significantly reduce ID8-Luc invasion (P < 0.01; t test with Welch's correction). Data are represented as mean  $\pm$  SEM of  $n = 6$ . Representative data are shown from at least three independent experiments. (B) ID8 cells were co-cultured with IKK $\beta$ -targeted BMDMs or TAMs and respective control cells. Fluorescence caspase 3/7 activity was assessed after 0, 3, and 6 h. Co-culture with IKK $\beta^{\text{DN}}$  and IKK $\beta^{\Delta}$  BMDMs or TAMs expressing IKK $\beta^{\text{DN}}$  (TAM $_{\text{DN}}$ ) significantly increases caspase 3/7 activity in ID8 cells (P < 0.01; t test with Welch's correction). Data are represented as mean  $\pm$  SD of  $n = 6$ . Representative data are shown from at least three independent experiments. (C) [ $^{111}\text{In}$ ]oxine release assay after a 24-h co-culture of [ $^{111}\text{In}$ ]-labeled ID8 cells with mock, IKK $\beta^{\text{DN}}$ , TAM $_{\text{DN}}$  or IKK $\beta^{\text{fl}}$  and IKK $\beta^{\Delta}$  macrophages. [ $^{111}\text{In}$ ]-release in cell-free culture supernatants was measured after 24 h with a scintillation counter. IKK $\beta$ -targeted macrophages promote increased cytotoxicity. Data are represented as mean of  $n = 3$ . Representative data are shown from at least three independent experiments.

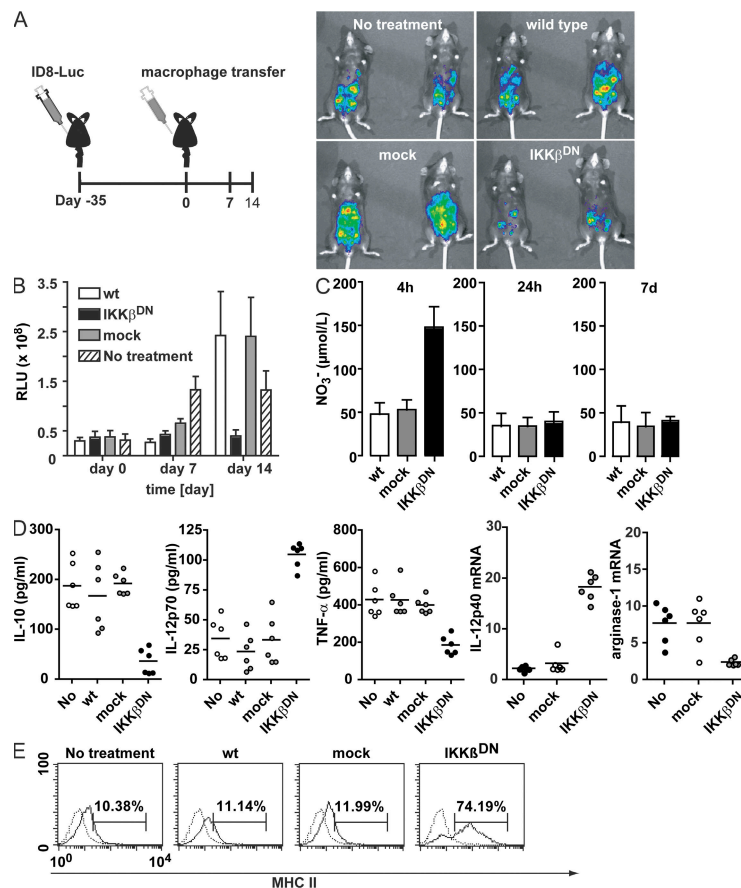
and elevated inducible nitric oxide (NO) synthase (NOS2) expression (Fig. 2 B and Fig. S1 C). Furthermore, increased NOS2 expression correlated with an increase in NO levels in the co-culture supernatant (Fig. 2 A and Fig. S1 B). These data suggested that inhibition of IKK $\beta$  signaling in BMDMs co-cultured with ID8 cells, and TAMs isolated from established tumors, switched macrophages from an M2 to a “classically” activated M1 phenotype.



**Figure 2. IKK $\beta$  maintains the alternative phenotype of tumor-polarized macrophages.** WT, mock, IKK $\beta$ <sup>DN</sup> or IKK $\beta$ <sup>fl/fl</sup>, and IKK $\beta$ <sup>Δ/Δ</sup> BMDMs were co-cultured with ID8 cells for 24 h. Supernatant was collected after 24 h and analyzed for IL-10, IL-12p70, TNF- $\alpha$ , and NO production by ELISA. (A) Co-cultured WT macrophages express an immunosuppressive IL-10<sup>high</sup> IL-12p70<sup>low</sup> TNF- $\alpha$ <sup>high</sup> profile. However, supernatant from co-cultured IKK $\beta$ -deleted macrophages show a significant decrease in IL-10 and TNF- $\alpha$  ( $P < 0.01$ ;  $t$  test with Welch's correction) but increase in IL-12p70 ( $P < 0.01$ ;  $t$  test with Welch's correction). Data are represented as mean  $\pm$  SD of  $n = 3$ . Representative data are shown from at least three independent experiments. (B) In parallel, total RNA was isolated for real-time PCR analysis of IL-12p40 and arginase-1 in co-cultured macrophages. Targeting IKK $\beta$  in macrophages switches their phenotype to an IL-12<sup>high</sup> arginase-1<sup>low</sup> NOS2<sup>high</sup> profile, consistent with an M1 phenotype. In addition, IKK $\beta$ -targeted macrophages secrete significantly higher levels of NO, measured as nitrite by Griess assay ( $P < 0.01$ ; Mann-Whitney test). Data are represented as mean  $\pm$  SD of  $n = 3$ . Representative data are shown from at least four independent experiments. (C) Macrophages were co-cultured with ID8 cells, and protein extracts were prepared at the indicated time points for biochemical analysis. Expression of NF- $\kappa$ B p65, serine 536 phosphorylation of p65, IKK $\beta$ , Stat1, tyrosine 701 phosphorylation of Stat1 (Tyr701), and NOS2 was measured by immunoblot analysis of cell lysates using  $\beta$ -actin as a loading control. Representative data are shown from at least three independent experiments. (D) [<sup>111</sup>In]oxine release assay after co-culture of ID8 ovarian cancer cells with IKK $\beta$ <sup>DN</sup> or IKK $\beta$ <sup>Δ/Δ</sup> BMDMs. IKK $\beta$ -targeted macrophages promote increased cytotoxicity that was reversed by the addition of the selective NOS2 inhibitor 1400W. Data are represented as mean of  $n = 3$ . Representative data are shown from at least three independent experiments.

As Stat1 is an essential transcription factor for IL-12, MHC class II, and NOS2 expression (13), we analyzed Stat1 activation in macrophages co-cultured with tumor cells. When IKK $\beta$ -targeted macrophages were co-cultured with ID8 tumor cells, they showed increased tyrosine phosphorylation of Stat1 (pY-Stat1, Tyr701), which correlated with increased NOS2 expression (Fig. 2 C). NOS2 has been directly implicated in macrophage-mediated tumoricidal activity (14), and several in vitro studies have demonstrated that NO donors are cytotoxic and proapoptotic to tumor cells (15, 16). We performed [<sup>111</sup>In]-release cytotoxicity assays in the presence of a selective NOS2 inhibitor, 1400W, and demonstrated that inhibition of NOS2 rescues the cytotoxic activity of IKK $\beta$ -targeted BMDMs or TAMs isolated from established tumors (Fig. 2 D and Fig. S1 D). These data suggest that IKK $\beta$ -mediated suppression of Stat1 activation inhibits the M1 phenotype and tumoricidal activity of tumor-polarized macrophages.

To investigate the in vivo implications of these observations, we adoptively transferred IKK $\beta$ -targeted macrophages into mice bearing established ovarian tumors to test if "re-educating" macrophages through IKK $\beta$  inhibition could affect tumor progression. ID8 ovarian cancer cells that expressed firefly luciferase were injected i.p. into mice. After 5 wk, these mice had malignant ascites and established tumors, with deposits throughout the peritoneum (Figs. 3 A and 4 A, and Fig. S2, which is available at <http://www.jem.org/cgi/content/full/jem.20080108/DC1>) (11). We then adoptively transferred  $5 \times 10^6$  WT-, IKK $\beta$ <sup>Δ/Δ</sup>-, or IKK $\beta$ <sup>DN</sup>-expressing BMDMs or TAMs isolated from established tumors and infected in vitro with the IKK $\beta$ <sup>DN</sup> adenovirus (TAM<sub>DN</sub>). Tumor progression was assessed in situ by bioluminescence imaging on days 0, 7, and 14 after adoptive transfer (Figs. 3 A and 4 A, and Fig. S2). Transfer of IKK $\beta$ -targeted BMDMs or TAMs into tumor-bearing mice led to a significant ( $P < 0.01$ ) decrease in tumor burden when compared with control macrophages (Figs. 3 B and 4 B, and Fig. S2). After 14 d, luciferase levels were still significantly lower in the IKK $\beta$ -targeted group compared with the untreated or control groups (Figs. 3 B and 4 B, and Fig. S2). We next investigated whether the increase in NOS2 expression in IKK $\beta$ -targeted macrophages was associated with enhanced tumoricidal activity in vivo. 4 h after adoptive transfer of IKK $\beta$ -targeted BMDMs or TAMs, there were significantly higher NO levels in the peritoneum compared with control groups (Figs. 3 C and 4 C, and Fig. S2;  $P < 0.01$ ); however, this was not sustained and NO levels were no different 24 h after transfer of macrophages, despite the sustained effect on tumor growth (Figs. 3 C and 4 C, and Fig. S2). To analyze the TAM phenotype in vivo, we measured IL-10, IL-12, and TNF- $\alpha$  levels in ascitic fluid 14 d after macrophage transfer (Figs. 3 D and 4 D, and Fig. S2). Mice receiving IKK $\beta$ -targeted macrophages showed a significant change in cytokine production with a switch to an IL-10<sup>low</sup> IL-12<sup>high</sup> and TNF- $\alpha$ <sup>low</sup> profile (Figs. 3 D and 4 D, and Fig. S2;  $P < 0.01$ ). CD11b<sup>+</sup> ascitic macrophages from these mice expressed increased IL-12p40 and decreased arginase-1 mRNA



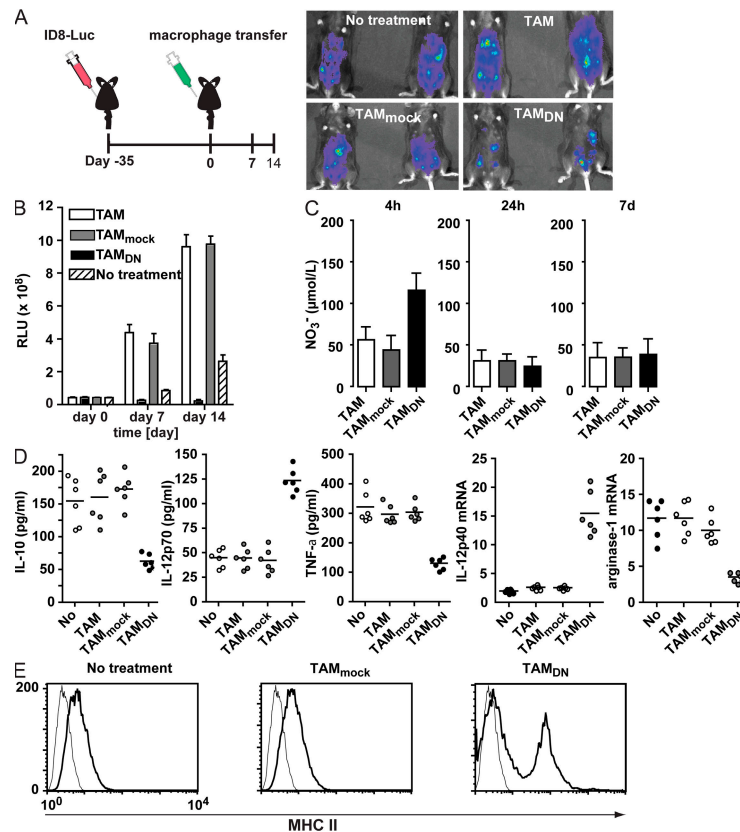
**Figure 3. Adoptive transfer of IKK $\beta$ -targeted BMDMs inhibits tumor growth in vivo.** (A) Representative bioluminescence imaging in vivo 14 d after adoptive transfer of BMDMs ( $n = 6$ ; three independent experiments). ID8-Luc were injected i.p. into syngeneic mice and tumors were allowed to develop. After 35 d, WT, mock, and IKK $\beta$ <sup>DN</sup> BMDMs were adoptively transferred and peritoneal cells were collected after an additional 7 and 14 d. Bioluminescence is presented as a pseudocolor scale: red, the highest photon flux; blue, the lowest photon flux. (B) Quantification of bioluminescence from primary tumors ( $n = 6$  each) obtained on days 0, 7, and 14. Adoptively transferred IKK $\beta$ <sup>DN</sup> BMDMs significantly reduced ID8 growth on day 14 ( $P < 0.05$ ;  $t$  test with Welch's correction). (C) NO secretion into the peritoneal cavity. IKK $\beta$ <sup>DN</sup> BMDMs increased NO release 4 h after adoptive transfer ( $P < 0.05$ ;  $t$  test with Welch's correction). Data are represented as mean  $\pm$  SD of  $n = 6$ . Representative data are shown from at least two independent experiments. (D) Cytokine profile in tumor ascites after 14 d. Ascites from mice treated with IKK $\beta$ -targeted BMDMs contained significantly lower amounts of IL-10 and TNF- $\alpha$  but higher amounts of IL-12 ( $P < 0.01$ ; Mann-Whitney test). Total RNA was isolated from CD11b<sup>+</sup>-selected ascitic macrophages for real-time PCR analysis of IL-12p40 and arginase-1 expression. Data are represented as fold induction of mRNA expression compared with WT macrophages ( $n = 6$ ). (E) MHC class II expression in the ascitic CD11b<sup>+</sup> myeloid cell population measured by FACS (percentage of positive cells indicated). Representative histograms are shown from  $n = 6$ .

(Figs. 3 D and 4 D, and Fig. S2) and showed higher MHC class II surface expression compared with control macrophages (Figs. 3 E and 4 E, and Fig. S2). These data show that targeting IKK $\beta$  activity in BMDMs prevents their polarization to an M2 phenotype by the tumor microenvironment in vivo. In addition, we show that inhibition of IKK $\beta$  in TAMs isolated from established tumors switches their phenotype from tumor-promoting M2 to antitumor M1.

Despite a sustained antitumor effect upon transfer of IKK $\beta$ -targeted macrophages in vivo, there was only a transient increase in NO production. This suggested that a different mechanism might be responsible for the long-term inhibition of tumor growth. Of particular interest was a significant ( $P < 0.01$ ) increase in NK cells (Fig. 5 A and Fig. S3,

which is available at <http://www.jem.org/cgi/content/full/jem.20080108/DC1>) in ascites from mice adoptively transferred with IKK $\beta$ -targeted macrophages. We therefore reasoned that an IL-12-mediated increase in NK cells in the peritoneum may contribute to the tumoricidal effect of targeting IKK $\beta$  in TAMs. To investigate this hypothesis, we treated mice with a neutralizing IL-12p40 antibody before adoptive transfer of macrophages. Neutralizing IL-12 in our model rescued the increased recruitment of NK cells in mice receiving IKK $\beta$ -targeted macrophages and restored tumor growth to similar levels as in controls (Fig. 5, A–C, and Fig. S3). The p40 subunit of IL-12 is shared with the related proinflammatory cytokine IL-23 (17). To rule out a role for IL-23, we used a neutralizing antibody against IL-23p19.





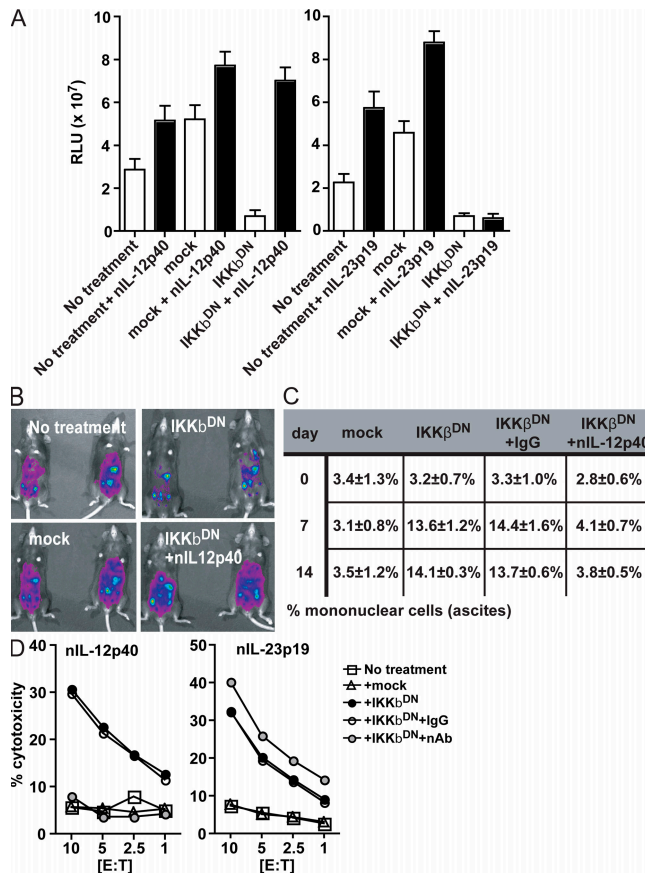
**Figure 4. Adoptive transfer of IKK $\beta$ -targeted TAMs inhibits tumor growth in vivo.** (A) Representative bioluminescence imaging in vivo 14 d after adoptive transfer of TAMs isolated from established ID8 tumors ( $n = 6$ ; three independent experiments). ID8-Luc were injected i.p. into syngeneic mice and tumors were allowed to develop. After 35 d, mock- and IKK $\beta^{\text{DN}}$ -infected TAMs (TAM<sub>mock</sub>, TAM<sub>DN</sub>, respectively) were adoptively transferred and peritoneal cells were collected after an additional 7 and 14 d. Bioluminescence is presented as a pseudocolor scale: red, the highest photon flux; blue, the lowest photon flux. (B) Quantification of bioluminescence from primary tumors ( $n = 6$  each) obtained on days 0, 7, and 14. Adoptive transfer of TAM<sub>DN</sub> significantly reduced ID8 growth on day 14 ( $P < 0.05$ ;  $t$  test with Welch's correction). (C) NO secretion into the peritoneal cavity. Transfer of TAM<sub>DN</sub> significantly increased NO production 4 h after adoptive transfer ( $P < 0.05$ ;  $t$  test with Welch's correction). Data are represented as mean  $\pm$  SD of  $n = 6$ . Representative data are shown from at least two independent experiments. (D) Cytokine profile in tumor ascites after 14 d. Ascitic fluid after transfer of TAM<sub>DN</sub> contained significantly lower amounts of IL-10 and TNF- $\alpha$  but higher amounts of IL-12 ( $P < 0.01$ ; Mann-Whitney test). Total RNA was isolated from CD11b<sup>+</sup>-selected TAMs for real-time PCR analysis of IL-12p40 and arginase-1 expression. Data are represented as fold induction of mRNA expression compared with TAM<sub>mock</sub>-treated mice ( $n = 6$ ). (E) MHC class II expression in the ascitic CD11b<sup>+</sup> TAMs measured by FACS (percentage of positive cells indicated). Representative histograms are shown from  $n = 6$ .

Neutralizing IL-23p19 had no effect on tumor growth (Fig. 5 C and Fig. S3). In further experiments, we recapitulated NK cell-mediated tumoricidal activity ex vivo using purified spleen NK cells. DX5<sup>+</sup> NK cells were isolated and treated with ascitic fluid from the adoptive transfer experiments described above. When NK cells were stimulated with ascites from mice receiving IKK $\beta$ -targeted macrophages, they showed a significant increase in tumoricidal activity (Fig. 5 D and Fig. S3). This increase in cytotoxicity was rescued with a neutralizing antibody to IL-12p40, whereas neutralizing IL-23p19 had an additive effect (Fig. 5 D and Fig. S3).

The data described above show that IKK $\beta$  signaling in macrophages maintains their alternative tumor-promoting phenotype. The experiments described in Fig. 1 show that IL-1R/MyD88 signaling in macrophages is important in driving tumor cell invasion, suggesting that IL-1 may be the soluble

mediator of NF- $\kappa$ B activation in our co-culture system. To investigate the role of IL-1R/MyD88 signaling in macrophage polarization in vivo, we adoptively transferred BMDMs deficient in MyD88, TLR2, TLR4, or IL-1R into ID8 tumor-bearing mice. Mice treated with IL-1R<sup>-/-</sup> or MyD88<sup>-/-</sup> macrophages had significantly less tumor burden after 14 d (S4A). In addition, ascitic fluid from mice treated with IL-1R<sup>-/-</sup> or MyD88<sup>-/-</sup> macrophages contained lower levels of IL-10 but higher levels of IL-12p70 compared with control groups (S4B). A similar switch toward an M1 phenotype was observed in the resident ascitic CD11b<sup>+</sup> population with an IL-12p40<sup>high</sup> arginase-1<sup>low</sup> expression profile (S4C).

In summary, these data show that inhibition of IKK $\beta$  activity specifically in TAMs is able to reverse their tumor-polarized phenotype and re-educate them to actively kill tumor cells by both direct tumoricidal activity through NO



**Figure 5. Increased IL-12-dependent NK cell recruitment.** (A) Neutralizing IL-12p40 but not IL-23p19 rescued the antitumor effect of adoptively transferred IKK $\beta$ DN BMDMs ( $P < 0.01$ ; Mann-Whitney test). Data are represented as mean  $\pm$  SEM of  $n = 6$ . Representative data are shown from two independent experiments. (B) Representative bioluminescence image of the IL-12p40-neutralizing experiment ( $n = 6$ ). (C) Adoptive transfer of IKK $\beta$ DN BMDMs increases NK cell recruitment in ID8 tumors ( $P < 0.01$ ; Mann-Whitney test). Data are represented as mean  $\pm$  SD of  $n = 6$ . Representative data are shown from two independent experiments. (D) Ex vivo NK cell cytotoxicity assay. Splenic DX5 $^{+}$ -enriched NK cells were stimulated with ascites from tumor-bearing mice, which had either no treatment or were treated with adoptive transfer of mock or IKK $\beta$ DN BMDMs. Ascites from mice treated by adoptive transfer of IKK $\beta$ DN BMDMs led to a significant increase in NK cell-mediated tumor cell cytotoxicity ( $P < 0.01$ ;  $t$  test with Welch's correction). The addition of a neutralizing antibody against IL-12p40 but not IL-23p19 or the respective control antibody rescued the increased tumoricidal effect. Data are represented as mean of  $n = 3$ . Representative data are shown from three independent experiments.

production and the promotion of NK cell-mediated antitumor immunity through IL-12.

The studies described above reveal a new role for IKK $\beta$  in cancer-related inflammation. We demonstrate a previously unknown function for IKK $\beta$  in maintaining the alternative phenotype of TAMs and negative regulation of macrophage tumoricidal activity. In malignant disease, macrophages are recruited to the tumor from the circulation and become polarized to an alternative immunosuppressive phenotype,

characterized by high expression of IL-10, TNF- $\alpha$ , and arginase-1, but low NOS2, IL-12, and MHC II expression. There is a large macrophage population in many tumors, and our data suggest that we may be able to harness their intrinsic anti-tumor potential to promote tumor regression. During the 1990s there were clinical trials of in vitro-activated autologous macrophages, using either IFN- $\gamma$  alone or in combination with LPS; however, this approach had only limited success (18, 19). We have shown convincingly that targeting IKK $\beta$  in TAMs reverses their tumor-promoting activity and can drive tumor regression in vivo. These studies suggest that IKK $\beta$  inhibitors could represent a powerful therapeutic tool in cancer therapy. Besides the direct effects on antiapoptotic signaling in malignant cells, IKK $\beta$  inhibition may also modify the inflammatory microenvironment to promote an antitumor immune response.

## MATERIALS AND METHODS

**Cell lines and reagents.** The mouse ovarian cancer cell line ID8 (provided by K. Roby, University of Kansas, Kansas City, KS) (20) was cultured as described previously (10). Lentiviral infection of ID8 cells with luciferase reporter construct was performed as described previously (21). In vitro luciferase activity was assayed in  $0.5 \times 10^6$  ID8 cells in triplicate and according to the manufacturer's instructions (Promega). ID8-macrophage co-cultures were performed as described previously (22).

**Generation of mouse macrophages.** Mouse macrophages were derived from the bone marrow of WT, TLR2, TLR4, MyD88, and IL-1R knock-out C57BL/6 mice or IKK $\beta$  floxed mice (IKK $\beta^{f/f}$ ) and cultured for 7 d in DMEM medium supplemented with 10 ng/ml recombinant mouse M-CSF (R&D Systems). TAMs were isolated from the peritoneal cavity of mice in which ID8 ovarian cancers had been growing i.p. for 10 wk.

**Macrophage infection.** BMDMs or CD11b $^{+}$ -selected in vivo-polarized TAMs were infected with recombinant adenovirus expressing a dominant-negative inhibitor of IKK $\beta$  (IKK $\beta$ DN, TAM $^{DN}$ ). These viruses are E1/E3 deleted, belong to the Ad5 serotype, and have been used in other studies (23, 24). IKK $\beta^{f/f}$  BMDMs were infected with recombinant adenovirus expressing Cre. IKK $\beta^{f/f}$  BMDMs infected with Adv-GFP were used as a control. Infections were performed at day 4 of macrophage differentiation in the presence of recombinant mouse M-CSF (R&D Systems).

**Western blotting.** Immunoblotting was performed with NOS2 (Santa Cruz Biotechnology, Inc.),  $\beta$ -actin (Sigma-Aldrich), NF- $\kappa$ B p65, phospho-NF- $\kappa$ B p65 (Ser536), Stat1, phospho-Stat1 (Tyr701), IKK $\beta$  and phospho-IKK $\beta$  (Ser181), and I $\kappa$ B $\alpha$  and phospho-I $\kappa$ B $\alpha$  (Ser32; all Cell Signaling Technology).

**DNA-binding activity of transcription factor NF- $\kappa$ B p50/p65.** NF- $\kappa$ B activation was measured using the TransFactor assay (Millipore) (11).

**Adoptive transfer of macrophages into mice bearing established tumors.** 8-wk-old C57BL/6 female mice (B&K Universal) were injected i.p. with  $10^6$  ID8-Luc cells, and tumors were allowed to establish for 35 d.  $5 \times 10^6$  macrophages were transferred adoptively i.p. on day 35. Neutralizing antibodies against IL-12p40/70 (17.8; R&D Systems), IL-23p19 (G23-8; eBioscience), or the respective control antibody were injected i.p. at 100  $\mu$ g/mouse twice weekly. To assess tumor progression, luciferase activity was measured in cell lysates. All mice were maintained in the Biological Services Unit, Institute of Cancer and the CR-UK Clinical Centre, Barts and The London School of Medicine, and used according to established institutional guidelines under the authority of a UK Home Office project license (Guidance on the Operation of Animals, Scientific Procedures Act 1986).

**Bioluminescence imaging.** Tumor progression was assessed in situ by bioluminescent imaging as described previously (21).

**Flow cytometry.** The phenotype of transferred macrophages was assessed by FACS analysis on a FACScan flow cytometer and analyzed using Cellquest software (Becton Dickinson).

**Cytotoxicity assays.** For  $^{111}\text{In}$ -release assay, a cytotoxicity assay was performed for 24 h with macrophages. Radioactivity was measured by counting 1 min in a  $\gamma$  counter. For  $^{51}\text{Cr}$ -release assays, NK cell cytotoxicity was defined as lysis of target cells after 4 h. Ascites samples from tumor-bearing mice treated with adoptive transfer of modified macrophages were concentrated under vacuum for 30 min at 37°C. In vitro stimulation with ascites was performed by adding 10  $\mu\text{g}/\text{ml}$  concentrated total ascites at the start of the 4-h cytotoxicity assay. Neutralizing antibodies against IL-12p40/70 (17.8; R&D Systems) or IL-23p19 (G23-8; eBioscience) or the respective control antibody were added to the release assay at 10  $\mu\text{g}/\text{ml}$ .

The percentage of cytotoxic activity was calculated as the specific release of radioactivity: % cytotoxicity = [(experimental isotope – spontaneous isotope)/total isotope – spontaneous isotope]  $\times$  100.

**ELISA-based assays.** To determine caspase-3/7 activity (Promega), cytokine levels in cell culture supernatant or ascite ELISAs (R&D Systems) were used according to the manufacturer's instructions.

**Statistical analysis.** Experiments were performed in triplicate, and representative data are shown. Results were tested for statistical significance using Student's *t* test with Welch's correction or the Mann-Whitney test with GraphPad Prism Version 4.0c software.

**Online supplemental material.** Fig. S1 shows the results of the NF- $\kappa\text{B}$  activity assay, the in vitro profile for in vivo-generated TAMs under coculture. Fig. S2 summarizes the in vivo adoptive transfer of IKK $\beta$  macrophages and their respective controls. Fig. S3 shows the effect of neutralizing IL12p40 and IL-23p19, and NK cell data. Fig. S4 shows ex vivo analysis of tumor burden in mice adoptively transferred with macrophages from MyD88, TLR2, TLR4, and IL-1R knockout mice. The online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20080108/DC1>.

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